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**Parallel assessment of the effects of bisphenol A and several of its analogs on the
adult human testis**

C. Desdoits-Lethimonier^{1,2}, L. Lesné^{1,2}, P. Gaudriault^{1,2}, D. Zalko³, J.P. Antignac⁴, Y. Deceuninck⁴, C. Platel⁵, N. Dejuqc-Rainsford^{1,2}, S. Mazaud-Guittot^{1,2}, B. Jégou^{1, 6*}

¹Inserm (Institut national de la santé et de la recherche médicale), Irset U1085, 9 Avenue du Professeur Léon Bernard, 35000 Rennes, CEDEX, France.

²Université de Rennes I, 9 Avenue du Professeur Léon Bernard, 35000 Rennes, CEDEX, France

³UMR 1331 TOXALIM, INRA (Institut National de la Recherche Agronomique), Chemin de Tournefeuille, Toulouse, France

⁴Oniris UMR INRA 1329 LABERCA,—La Chantrerie, Nantes, France

⁵CHU Rennes, Unité de coordination hospitalière des prélèvements d'organes et de tissus, 2 rue Henri Guilloux 35000 Rennes, France.

⁶EHESP—School of Public Health, 9 Avenue du Professeur Léon Bernard, 35000 Rennes, France

**Corresponding author: bernard.jegou@inserm.fr. Irset-Inserm U1085, 9 avenue du Professeur Léon Bernard, 35000 Rennes. Phone : +33 2 23 23 61 25 Fax: +33 2 23 23 50 55*

Running Title: Effects of bisphenol A and its analogs on adult human testis

Abstract

STUDY QUESTION: Are Bisphenol A (BPA) and BPA analogs (BPA-A) safe for male human reproductive function?

SUMMARY ANSWER: The endocrine function of human testes explants [assessed by measuring testosterone and insulin-like factor 3 (INSL3)] was impacted by exposure of the human adult testis explants to BPA/BPA-A.

WHAT IS KNOWN ALREADY: The few epidemiologic studies performed suggest that bisphenols have potential endocrine disruptive properties, but they did not identify clear and direct patterns of endocrine disruption.

STUDY DESIGN, SIZE, DURATION: Adult human testis explants in culture were exposed to BPA and the analogs bisphenol F (BPF), bisphenol S (BPS), bisphenol E (BPE), bisphenol B (BPB), and bisphenol A diglycidyl ether (BADGE) at 10^{-9} to 10^{-5} M for 24 h or 48h.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human adult testes were obtained from prostate cancer patients who had no hormone therapy, or from multiorgan donors. After ex vivo exposure to the investigated bisphenols, the measured outcomes were related to histopathology (gross morphology and germ cell viability determined by anti-caspase 3 immunohistochemistry), and the levels of testosterone, insulin-like factor 3 and inhibin B were measured using immunoassays. The levels of mRNA encoding key enzymes of bisphenol biotransformation were investigated by quantitative PCR: UGT2B15 UDP (glucuronosyltransferase two family, polypeptide B15), GUSB (glucuronidase beta), SULT1A1 and 3 (sulfotransferase family 1 A member 1 and 3) and STS (steroid sulfatase).

MAIN RESULTS AND THE ROLE OF CHANCE: A significant dose-dependent inhibition was found between testosterone levels measured in the culture medium and concentrations of BPA ($p=0.00778$ at 24 h and $p=0.0291$ at 48 h), BPE ($p=0.039$), and BPF ($p=0.00663$). The observed BPA and BPA-A-induced inhibition of testosterone production varied according to

duration of exposure and BPA/BPA-A concentrations. . BPA (10^{-9} M; $p<0.05$), BPB (10^{-9} M; $p<0.05$), BPS (10^{-9} and 10^{-8} M; $p<0.05$) and BADGE (10^{-5} M; $p<0.05$) were able to increase Leydig cell INSL3 production. By contrast, BPE dose dependently inhibited the latter hormone ($p=0.0372$). Conversely, Sertoli cell function (inhibin B) and germ cell viability were not significantly affected by either bisphenols.

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: Environmental compounds cannot be deliberately administered to men, justifying the use of an *ex vivo* approach. A relatively low number of testes samples were available for analysis ($n=3$, except for testosterone secretion with $n=5$). The active concentrations of BPA and BPA-A used in the study were higher than those found in human biological fluids.

WIDER IMPLICATIONS OF THE FINDINGS: Under our experimental conditions, direct exposure to BPA or BPA-A can result in endocrine disturbance in the adult human testis.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by Inserm (Institut National de la Santé et de la Recherche Médicale), EHESP - School of Public Health, University of Rennes1, by grants from the Agence Nationale de la Recherche (ANR; grant#ANR-13-CESA-0012-03 NEWPLAST), and Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES; grant#EST-2010/2/046 (BPATESTIS)). All authors declare they have no current or potential competing financial interests.

Key words: Bisphenols ; anti-androgenic compounds ; reproductive function ; endocrine disruptor; human testis; Leydig cells; Sertoli cells; testosterone; insulin-like factor 3; inhibin B.

Introduction

Bisphenol A (BPA) (2,2-bis (4-hydroxyphenyl) propane; CAS # 80 05-7) is currently produced in huge quantities (Vandenberg *et al.*, 2007). The population may be exposed to BPA from fetal life to adulthood (Cobellis *et al.*, 2009; Vandenberg *et al.*, 2010; Braun and Hauser, 2011; Braun *et al.*, 2011; Koch *et al.*, 2012). Many reports claim that it belongs to the growing list of so-called endocrine-disrupting chemicals (EDC; Beronius *et al.*, 2010; Teeguarden and Hanson-Drury, 2013). Decision of a number of regulatory agencies to ban BPA from food packaging has led to the gradual development of a number of bisphenol analogs (BPA-A) to replace BPA in several applications. Among them, bisphenol S (BPS), bisphenol F (BPF), bisphenol E (BPE), bisphenol B (BPB), and bisphenol A diglycidyl ether (BADGE) have replaced BPA for use in thermal receipt paper (Liao *et al.*, 2012a) or epoxy resin products (Danzl *et al.*, 2009). BPA is known to migrate from packaging materials into foodstuffs (Muncke, 2009; Coulier *et al.*, 2010; Deceuninck *et al.*, 2014; Oldring *et al.*, 2014; Sungur *et al.*, 2014). BPA and BPF have also been found in a high percentage of food samples tested. Canned foods contain the highest concentrations of bisphenols (Liao and Kannan, 2014). Furthermore, BPA-A have been reported in various environmental matrices, including water, sediment and dust (Fromme *et al.*, 2002; Liao *et al.*, 2012b; Song *et al.*, 2012; Lee *et al.*, 2014) .

BPB, BPS, BPF, BPA, and BPAF have been detected in urine samples from some individuals (Cunha and Fernandes, 2010; Yang *et al.*, 2014). BADGE and its hydrated derivatives were detected in human adipose fat samples (Wang *et al.*, 2015).

We investigated here for the first time the direct effects of BPA and BPA-A substitutes on the physiology of the human adult testis using an organotypic culture model.

Materials and Methods

Ethics statement

Adult testes were obtained from prostate cancer patients who had no anti-androgen treatment or multiorgan donors (all donors considered mean age: 46.75; SEM: \pm 4.65 years). The local ethics committee approved the protocol, and written informed consent was obtained from either donors or their next of kin (CCPPRB Rennes; authorization 05/39-566; Agence de la Biomédecine; authorization #PFS09-015).

The TEstis EXplant ASsay (TEXAS):

The testes obtained from patients or donors were placed at 4°C and processed immediately. The observation by transillumination allowed us to ascertain that the testes displayed spermatogenesis (Roulet *et al.*, 2006). Four 3-mm³ testis explants were placed onto a PET insert (Falcon Labware; Becton Dickinson, Lincoln Park, NJ, USA) at the interface of air in 1 mL of Dulbecco's Modified Eagle's Medium supplemented with antibiotics, 1 mM sodium pyruvate, 4 mM glutamine, 100 ng/ml of vitamin A, 200 ng/ml of vitamin E, 50 ng/ml of vitamin C, 10 µg/ml of insulin and 5 µg/ml of transferrin and with 1 IU/ml hCG for culture, in 12-well plates. For the exposure experiments the media contained either 0.1% dimethylsulphoxide (DMSO) as a control, or BPA, BPB, BPE, BPF, BPS, or BADGE at different concentrations (purity > 99%; Sigma-Aldrich, Saint Quentin Fallavier, France, diluted in DMSO at a final concentration of 0.1%). Four wells corresponding to the replicates performed for each independent experiment were analyzed for each condition. The exposures lasted 24 and 48 hours, with a total medium change at 24 h. Media were stored at -80°C. On the day of collection, 3 explants for each culture condition were collected at random, fixed in either neutral buffered 4% formaldehyde or Bouin's fixative and embedded in paraffin. Then they were sliced into 5.0 µm-thick sections, and stored at + 4°C until immunostaining. The potential external (procedural) contamination induced by BPA present in the plastic or the medium was checked by measuring the amount of BPA before and after treatment by gas chromatography coupled to tandem mass spectrometry GC-MS/MS (Limit of quantification (LOQ) = 3×10^{-10} M;

Deceuninck *et al.*, 2014). The expected BPA concentrations were consistent with the concentrations actually measured. BPA biotransformation was studied by high-performance liquid chromatography (HPLC) coupled to an online radioactivity detection system using [³H]-BPA as already described (Ben-Maamar *et al.*, 2015).

Immunostaining

Leydig cells were labeled with a rabbit primary antibody directed against the cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) (1/250; Sigma Aldrich), on formaldehyde-fixed tissue. The apoptotic cells were stained with the primary rabbit antibody directed against cleaved caspase-3 (1/100; Ozyme, France; Desdoits-Lethimonier *et al.*, 2012). Slides were scanned with a NanoZoomer slide scanner (Hamamatsu Photonics, France, at *Plateforme H2P2*, Biosit, Rennes, France). Caspase-3 positive cells were counted with ImageJ free software.

Hormone production

Testosterone was assayed in the media with a specific radioimmunoassay (RIA; Immunotech, Beckman Coulter, France). The intra- and inter-assay coefficients of variation were ≤ 8.6 and 11.9%, respectively. Control testis explants produced an average of 10.26, SEM ± 2.50 ng/ml/explant testosterone after 24 h of culture, and 8.47 ± 2.16 ng/ml/explant after 48 h. Insulin-like factor 3 (INSL3) production was measured by RIA (RK-035-27, Phoenix France). The intra- and inter-assay coefficients of variation were ≤ 15 and 7%, respectively, and the lower limit of detection was 20.17 pg/ml. Control testis explants produced an average of 620 ± 103 pg/ml/explant INSL3 after 24 h of culture, and 446 ± 99 pg/ml/explant after 48 h. Inhibin B was assayed by ELISA (DSL-10-84100 Active, Beckman Coulter, France). The intra- and inter-assay coefficients of variation for serum samples were ≤ 5.6 and 7.6%, respectively. Control

testis explants produced an average of 570.43 ± 53.2 pg/ml/explant inhibin B after 24 h of culture, and 529.44 ± 55 pg/ml/explant after 48 h.

Quantitative PCR (qPCR)

RNA was extracted from human testes and liver samples with a NucleoSpin RNA II kit (Macherey-Nagel, France). Total RNAs (250 ng) were reverse transcribed with the Iscript cDNA Synthesis Kit (Biorad; France). Quantitative PCR was performed with the iTaq Universal SYBR Green Supermix (Biorad, France) with a 2.5 μ l cDNA template in a CFX384 Touch Real-Time PCR Detection System (Biorad). The following amplification program was used: an initial denaturation for 3 min at 95°C; 40 cycles of 10 sec denaturation at 95°C; and 30 sec at 62°C for annealing and extension. Dissociation curves were produced with a thermal melting profile performed after the last PCR cycle. To avoid amplification of contaminating genomic DNA, primer pairs were selected on 2 different exons (for genes studied, see Table 1). Basic leucine zipper and W2 domains 1 (BZW1) and GAPDH mRNA were used as internal controls for normalization. Results were calculated by the $\Delta\Delta$ CT method as n-fold differences in target gene expression with respect to the reference gene and the calibration sample.

Statistics

Data were analyzed with GraphPad prism v6.05 (GraphPad software, USA). Values are expressed as the mean \pm SEM. Differences between BPA, BPA-A treated and the corresponding control were analyzed using the non-parametric Mann-Whitney test. Dose-response relationships were analyzed using a non-parametric Spearman correlation test, and slopes and P values were indicated when significant.

Results

Biotransformation of BPA

Radio-HPLC analysis of the biotransformation of human testicular explants revealed that after 24 h of incubation with H³-BPA, regardless of dose, only parent BPA was recovered in either the medium or the testis extracts, indicating that no or very little biotransformation of BPA took place. We also demonstrate that the testes expressed mRNAs for the main enzymes necessary to biotransform BPA, specifically UDP glucuronosyltransferase 2 family, polypeptide B15 (UGT2B15), glucuronidase beta (GUSB), sulfotransferase family 1A member 1 (SULT1A1), sulfotransferase family 1A member (SULT1A3), and steroid sulfatase (STS) (Figure 1). UGT2B15 mRNA was very weakly expressed in the testis compared with the liver (0.1%). The testicular mRNA levels of GusB, Sult1A1, and Sult1A3 reached 36.1%, 16.4%, and 27.2% of those in the liver, respectively. Sult1A3 and STS were expressed in about the same range in both the testis and the liver.

Histopathology

Neither BPA nor the BPA-A impaired the histology of the testis explants according to staining of cleaved caspase-3 (1.57×10^{-4} positive caspase-3 cells/ μm^2 in control explants; Figure 2) and CYP11A1 (data not shown).

Testosterone

Testosterone levels decreased significantly and dose-dependently after BPA exposure ($\beta = -0.478$, $p = 0.00778$ at 24 h and $\beta = -0.399$, $p = 0.0291$ at 48 h; Figure 3A, B). BPA appeared most anti-androgenic at a dose of 10^{-5} M, at both time periods measured (24 and 48 h; -28.7% and -39.2% compared to control, respectively). Similarly, as levels of BPE at 24 h and BPF at 48 h rose, testosterone production fell significantly ($\beta = -0.424$, $p = 0.039$ and $\beta = -0.54$, $p = 0.00663$, respectively, Figure 3C, 3F). At 24 h, BPE induced a significant decrease at the highest dose (-37.4%, $p = 0.028$; Figure 3C). The response to BPF was more sensitive, as a

significant decrease in testosterone was observed at 10^{-6} M after 24 h and at 10^{-6} and 10^{-5} M after 48 h of exposure (Figure 3E, 3F).

BPB significantly decreased testosterone levels at 10^{-7} M after 24 h (-17%, $p=0.028$; Figure 3G) and 48 h (-47%, $p=0.028$; Figure 3H). BADGE significantly decreased testosterone levels by 12.3% at 10^{-9} M and 28.8% at 10^{-7} M after 24 h and by 19.1% after 48 h of culture with 10^{-8} M (Figure 3I, 3J).

The effects of BPS varied, with paradoxical trends at 24 h and 48 h. At the 24-h collection, testosterone levels had increased significantly at 10^{-6} M (+17.8%, $p<0.05$), but was significantly inhibited at a 10^{-8} M concentration at 48 h (-18.9%; $p=0.052$). The increases at 24 h for concentrations of 10^{-7} M and 10^{-6} M were attenuated at 48 h (12% and 16.6%, respectively; Figure 3K, 3L).

INSL3

BPA, BPB, and BPS significantly increased INSL3 production by Leydig cells at a low doses: 10^{-9} M for BPA and BPB after 24 h of exposure (Figure 4A, 4G), and 10^{-9} M and 10^{-8} M after 48 h of BPS exposure (Figure 4J). BPB tended to increase INSL3 levels consistently after 24 h and 48 h of treatment at 10^{-6} and 10^{-5} M. BADGE showed a significant dose-response capacity to increase INSL3 after 48 h of exposure; the highest dose yielded a significant increase of 31.5% (Figure 4L). By contrast, the exposure to BPE dose-dependently inhibited INSL3 levels (Figure 4C, D). After 48 h, a significant negative correlation was observed: as BPE increased, INSL3 levels decreased ($\beta=-0.814$, $p=2 \cdot 10^{-7}$; Figure 4D).

Inhibin B

Neither BPA nor any of the BPA-A significantly affected the production of inhibin B by Sertoli cells (Figure 5).

Discussion

Based on the use of an *ex-vivo* culture system previously used to study the effects of phthalates (Desdoits-Lethimonier *et al.* 2012), and of several analgesics (Albert *et al.* 2013), our data reveal that BPA and of BPA-A exhibit anti-androgenic properties in the adult human testis. The individual concentrations at which the effects on the Leydig cell hormones were observed varied according to the bisphenol studied, the duration of incubation, and the dose considered. Furthermore effects of BPA, BPB, BPS and BADGE (increases), as well as of BPE (decrease) were observed on INSL3 production.

The highest levels of urinary BPA have been associated with elevated estrogen levels in men (Kim *et al.*, 2014; Lassen *et al.*, 2014), as well as with elevated concentrations of serum LH and testosterone (Galloway *et al.*, 2010; Lassen *et al.*, 2014). Urinary BPA levels in BPA-exposed workers, compared to non-exposed workers have also been associated with significantly higher risks of male sexual dysfunction (Li *et al.*, 2010), decreased semen quality (Li *et al.*, 2011), and decreased androgen levels (Zhou *et al.*, 2013). It has also been shown that BADGE generates BPA endogenously in BADGE-exposed male workers and that their plasma FSH levels decrease as their BPA levels rise. Nonetheless, neither free testosterone nor LH levels are affected in these conditions (Hanaoka *et al.*, 2002). In male partners of pregnant women, urinary BPA levels have been inversely associated only with the free androgen index (FAI: testosterone/SHBG; sex hormone-binding globulin; Mendiola *et al.*, 2010). In men attending fertility clinics, urinary BPA concentrations are associated with a decline in semen quality parameters (Meeker *et al.*, 2010b; Knez *et al.*, 2014), and appear to influence inhibin B, FSH, and the estradiol/testosterone ratio (Meeker *et al.*, 2010a). Overall, only few epidemiological studies have been performed on the possible link between BPA and the endocrine balance in men, while no clear pattern of endocrine disruption has been demonstrated.

A global observation of the pattern of hormonal profiles obtained here indicates that BPA and BPA-A can generate non-monotonic dose-response relationships (NMDR), a feature frequently associated with BPA action (for reviews: Vandenberg, 2014; Lagarde *et al.*, 2015). In the present study, quantified dose-dependent curves were only found for BPA, BPF, and BPE. We did demonstrate that BPA and all the investigated BPA-A can exhibit anti-androgenic properties in the adult human testis, with the individual concentrations at which the bisphenols suppressed testosterone varying according to the bisphenol studied, duration of incubation, and dose considered. BPA still displayed some anti-androgenic effects after 24 h of exposure, which is a likely a consequence of the very low expression of UGT2B15 in the testis, as shown here. This is consistent with the expression of human UGT isoforms in 23 human tissues evidenced by others (Ohno and Nakajin, 2009). Like BPA, BPE significantly inhibited testosterone production at 10^{-5} M and at a rate close to that of BPA (35.4% for BPE and 27.5% for BPA) after 24 h of exposure. However, the effect of BPE was less persistent than that of BPA. As different enzymes are responsible for the hydroxylation of the different bisphenols (Schmidt *et al.*, 2013), BPE may be biotransformed more efficiently than BPA in the human testis. In our study, a lower concentration of BPF (10^{-6} M) had an inhibitory dose effect on testosterone production. This BPA-A, which is not approved as a food contact material in the European Union (EU), has been detected in beverages and canned food samples (Liao and Kannan, 2014; Yang *et al.*, 2014). In commercial milk samples, BPF was the bisphenol detected most frequently, followed by BPA and BPB (Grumetto *et al.*, 2013). BADGE and BPS are approved as food contact materials in the EU. In our human culture system, of all bisphenols tested, BADGE was the one that significantly displayed its anti-androgenic properties at the lowest dose (10^{-9} M), while BPS was pro-androgenic at 24h and anti-androgenic at 48h.

Before this study, the anti-androgenic properties of BPA had been described in various animal experiments (Kawai *et al.*, 2003; Kabuto *et al.*, 2004; Tanaka *et al.*, 2006), in the human adrenocortical NCI-H295R cell line (Zhang *et al.*, 2011), and in human and rat microsomes (Ye

et al., 2011). Like BPA, some BPA-A were also shown to display anti-androgenicity in the steroidogenic NCI-H295R cell line (Rosenmai *et al.*, 2014; Goldinger *et al.*, 2015). BPA and BPE, in the range of concentrations used in the present study, had inhibitory effects on testosterone similar to those in the human testis. In contrast, BPF inhibited testosterone production only weakly in the NCI-H295R cell line, and inhibition by BPS was slight (Rosenmai *et al.*, 2014; Goldinger *et al.*, 2015), consistent with the result in our system after 24 h of exposure. While BPB suppressed testosterone production here (at 10^{-7} M at both 24 and 48 h), its anti-androgenicity was close to that of BPA in the NCI-H295R cell line (Rosenmai *et al.*, 2014). Further support for our findings comes from a study that used the mouse Leydig cell line, known as the MA-10 cell line (Roelofs *et al.*, 2015).

The interindividual variations in the levels of INSL3, the other Leydig cell hormone investigated, were greater than those of testosterone, as generally observed (Ivell *et al.*, 2013; Mazaud-Guittot *et al.*, 2013). This and the NMDR also often encountered with this hormone complicated the interpretation of our data. Nevertheless it appears that, in contrast to testosterone, the levels of INSL3 generally tended to increase in the presence of BPA and BPA-A. The exception was for BPE, which dose-dependently reduced INSL3 levels after 48 h of exposure.

Our findings also indicate that inhibin B was not affected by exposure to BPA or its analogs. The epidemiological studies investigating a link between male BPA exposure and inhibin B levels are far from providing a clear picture. Urinary BPA was found to be inversely associated with inhibin B levels measured in the serum of the male partners in subfertile couples (Meeker *et al.*, 2011). Furthermore, male workers in the highest quartile of BPA measurements had lower inhibin B levels (Liu *et al.*, 2015). Nonetheless, no such correlation between BPA and inhibin B was observed among male partners of pregnant women (Mendiola

et al., 2010) or in Chinese male workers exposed or not exposed to BPA in factories (Zhuang *et al.*, 2015).

The mean concentration of BPA in human biological fluids (blood, urine, saliva) is generally found to be in the 10^{-9} M order of magnitude (for reviews : Calafat *et al.*, 2008; Bushnik *et al.*, 2010; Vandenberg *et al.*, 2010; Andra *et al.*, 2015). Therefore, the dose of 10^{-5} M of BPA, which was found to significantly inhibit testosterone here, was much higher than the concentrations recorded in the body fluids. The significant BPA-induced anti-androgenicity observed at 10^{-5} M in adult human testes was consistent with that obtained with human fetal testes when cultured in the presence of trophic hormone (hCG or LH), which optimizes the organ's integrity (histology and hormone production) during the culture periods investigated both in terms of histology (Ben Maamar *et al.*, 2015) and hormone production (Ben-Maamar *et al.*, 2015; Eladak *et al.*, 2015). The fact that BPA or BPA-A can impact the production of testosterone by the human fetal testis at doses lower than 10^{-5} M only appeared in the absence of trophic hormones (N'Tumba-Byn *et al.*, 2012; Ben-Maamar *et al.*, 2015; Eladak *et al.*, 2015). According to the human exposure studies available on BPA-A, equivalent values to those of BPA were found in urine for BPS and BPF, at average levels of about 10^{-9} - 10^{-10} M, (Zhou *et al.*, 2014). BPB and BADGE were detected infrequently and their levels were around 10^{-9} M (Cobellis *et al.*, 2009; Cunha and Fernandes, 2010; Wang *et al.*, 2012, 2015; Asimakopoulos *et al.*, 2014). The significant BPA-A-induced suppression of testosterone found here occurred for exposure at lower concentrations (generally 10^{-6} – 10^{-8} M) than that observed for BPA. In any case these lower active doses still represent higher doses than those found in the human body fluids. Whether or not the short time frame of our culture conditions, dictated by our concern to achieve optimal experimental conditions in terms of morphological quality of the explants, is an obstacle for demonstrating BPA/BPA-A-induced anti-androgenic effects for doses close to those of human exposure is unknown.

Whether the BADGE-induced anti-androgenic effect or the BPA and BPB-induced pro-INSL3 effects detected at 10^{-9} M represents an intrinsic effect of these BP compounds or corresponds to an effect resulting from the mixture of BADGE or of BPA and BPB with the low levels of BPA contaminating the control culture media is not known.

To conclude, based on the available epidemiological evidence (Minguez-Alarcon *et al.*, 2016) and the results of this series of experiments, further research is needed to clarify the actual risk of exposure to BPA and BPA-A on male and couple reproductive health.

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Authors' roles

B.J., C.D.L. and L.L. designed the research; C.D.L., L.L. and Y.D. performed the research, analysis and manuscript drafting; C.P. supervised orchidectomies; D.Z. supervised and performed the biotransformation capabilities of BPA. N.D.R., P.G. and S.M.G. contributed to critical discussion. J.P.A. contributed to critical discussion and coordinator of funding project. All authors contributed to critically reviewing the draft manuscript.

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Conflict of interest

All authors declare they have no current or potential competing financial interests.

References

- Andra SS, Charisiadis P, Arora M, Vliet-Ostaptchouk J V van, Makris KC. Biomonitoring of human exposures to chlorinated derivatives and structural analogs of bisphenol A. *Environ Int* 2015;**85**:352–379.
- Asimakopoulos AG, Thomaidis NS, Kannan K. Widespread occurrence of bisphenol A diglycidyl ethers, p-hydroxybenzoic acid esters (parabens), benzophenone type-UV filters, triclosan, and triclocarban in human urine from Athens, Greece. *Sci Total Env* [Internet] 2014;**470–471**:1243–1249.
- Ben-Maamar M, Lesne L, Desdoits-Lethimonier C, Coiffec I, Lassurguere J, Lavoue V, Deceuninck Y, Antignac JP, Bizec B Le, Perdu E, *et al.* An investigation of the endocrine-disruptive effects of bisphenol a in human and rat fetal testes. *PLoS One* [Internet] 2015;**10**:e0117226.
- Beronius A, Rudén C, Håkansson H, Hanberg A. Risk to all or none? *Reprod Toxicol* [Internet] 2010;**29**:132–146.
- Braun JM, Hauser R. Bisphenol A and children’s health. *Curr Opin Pediatr* [Internet] 2011;**23**:233–239.
- Braun JM, Kalkbrenner AE, Calafat AM, Yolton K, Ye X, Dietrich KN, Lanphear BP. Impact of Early-Life Bisphenol A Exposure on Behavior and Executive Function in Children. *Pediatrics* [Internet] 2011;**128**:873–882.
- Bushnik T, Haines D, Levallois P, Levesque J, Oostdam J Van, Viau C. Lead and bisphenol A concentrations in the Canadian population. *Heal Rep* [Internet] 2010;**21**:7–18.

- Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. *Env Heal Perspect* [Internet] 2008;**116**:39–44.
- Cobellis L, Colacurci N, Trabucco E, Carpentiero C, Grumetto L. Measurement of bisphenol A and bisphenol B levels in human blood sera from healthy and endometriotic women. *Biomed Chromatogr* [Internet] 2009;**23**:1186–1190.
- Coulier L, Bradley EL, Bas RC, Verhoeckx KC, Driffield M, Harmer N, Castle L. Analysis of reaction products of food contaminants and ingredients: bisphenol A diglycidyl ether (BADGE) in canned foods. *J Agric Food Chem* [Internet] 2010;**58**:4873–4882.
- Cunha SC, Fernandes JO. Quantification of free and total bisphenol A and bisphenol B in human urine by dispersive liquid-liquid microextraction (DLLME) and heart-cutting multidimensional gas chromatography-mass spectrometry (MD-GC/MS). *Talanta* [Internet] 2010;**83**:117–125.
- Danzl E, Sei K, Soda S, Ike M, Fujita M. Biodegradation of bisphenol A, bisphenol F and bisphenol S in seawater. *Int J Env Res Public Heal* [Internet] 2009;**6**:1472–1484.
- Deceuninck Y, Bichon E, Durand S, Bemrah N, Zendong Z, Morvan ML, Marchand P, Dervilly-Pinel G, Antignac JP, Leblanc JC, *et al.* Development and validation of a specific and sensitive gas chromatography tandem mass spectrometry method for the determination of bisphenol A residues in a large set of food items. *J Chromatogr A* [Internet] 2014;**1362**:241–249.
- Desdoits-Lethimonier C, Albert O, Bizec B Le, Perdu E, Zalko D, Courant F, Lesne L, Guille F, Dejuqc-Rainsford N, Jegou B. Human testis steroidogenesis is inhibited by phthalates. *Hum Reprod* [Internet] 2012;**27**:1451–1459.
- Eladak S, Grisin T, Moison D, Guerquin M, N'Tumba-Byn T, Pozzi-Gaudin S, Benachi A,

- Livera G, Rouiller-Fabre V, Habert R. A new chapter in the bisphenol A story: bisphenol S and bisphenol F are not safe alternatives to this compound. *Fertil Steril* [Internet] 2015;**103**:11–21.
- Fromme H, Kuchler T, Otto T, Pilz K, Muller J, Wenzel A. Occurrence of phthalates and bisphenol A and F in the environment. *Water Res* [Internet] 2002;**36**:1429–1438.
- Galloway T, Cipelli R, Guralnik J, Ferrucci L, Bandinelli S, Corsi AM, Money C, McCormack P, Melzer D. Daily bisphenol A excretion and associations with sex hormone concentrations: results from the InCHIANTI adult population study. *Env Heal Perspect* [Internet] 2010;**118**:1603–1608.
- Goldinger DM, Demierre AL, Zoller O, Rupp H, Reinhard H, Magnin R, Becker TW, Bourqui-Pittet M. Endocrine activity of alternatives to BPA found in thermal paper in Switzerland. *Regul Toxicol Pharmacol* [Internet] 2015;**71**:453–462.
- Grumetto L, Gennari O, Montesano D, Ferracane R, Ritieni A, Albrizio S, Barbato F. Determination of five bisphenols in commercial milk samples by liquid chromatography coupled to fluorescence detection. *J Food Prot* [Internet] 2013;**76**:1590–1596.
- Hanaoka T, Kawamura N, Hara K, Tsugane S. Urinary bisphenol A and plasma hormone concentrations in male workers exposed to bisphenol A diglycidyl ether and mixed organic solvents. *Occup Env Med* [Internet] 2002;**59**:625–628.
- Ivell R, Wade JD, Anand-Ivell R. INSL3 as a biomarker of Leydig cell functionality. *Biol Reprod* [Internet] 2013;**88**:147.
- Kabuto H, Amakawa M, Shishibori T. Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. *Life Sciences* 2004;**30**:2931–2940.
- Kawai K, Nozaki T, Nishikata H, Aou S, Takii M, Kubo C. Aggressive behavior and serum

testosterone concentration during the maturation process of male mice: the effects of fetal exposure to bisphenol A. *Environ Health Perspect* 2003;**111**:175–178.

Kim EJ, Lee D, Chung BC, Pyo H, Lee J. Association between urinary levels of bisphenol-A and estrogen metabolism in Korean adults. *Sci Total Env* [Internet] 2014;**470–471**:1401–1407.

Knez J, Kranvogel R, Breznik BP, Voncina E, Vlaisavljevic V. Are urinary bisphenol A levels in men related to semen quality and embryo development after medically assisted reproduction? *Fertil Steril* [Internet] 2014;**101**:215–221 e5.

Koch HM, Kolossa-Gehring M, Schröter-Kermani C, Angerer J, Brüning T. Bisphenol A in 24 h urine and plasma samples of the German Environmental Specimen Bank from 1995 to 2009: A retrospective exposure evaluation. *J Expo Sci Environ Epidemiol* [Internet] 2012;**22**:610–616.

Lagarde F, Beausoleil C, Belcher SM, Belzunces LP, Emond C, Guerbet M, Rousselle C. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. *Environ Heal* 2015;**14**:13.

Lassen TH, Frederiksen H, Jensen TK, Petersen JH, Joensen UN, Main KM, Skakkebaek NE, Juul A, Jorgensen N, Andersson AM. Urinary bisphenol A levels in young men: association with reproductive hormones and semen quality. *Env Heal Perspect* [Internet] 2014;**122**:478–484.

Lee S, Liao C, Song GJ, Ra K, Kannan K, Moon HB. Emission of bisphenol analogues including bisphenol A and bisphenol F from wastewater treatment plants in Korea. *Chemosphere* [Internet] 2014;**119C**:1000–1006.

Li D, Zhou Z, Qing D, He Y, Wu T, Miao M, Wang J, Weng X, Ferber JR, Herrinton LJ, *et al.* Occupational exposure to bisphenol-A (BPA) and the risk of self-reported male sexual

dysfunction. *Hum Reprod* [Internet] 2010;**25**:519–527.

Li DK, Zhou Z, Miao M, He Y, Wang J, Ferber J, Herrinton LJ, Gao E, Yuan W. Urine bisphenol-A (BPA) level in relation to semen quality. *Fertil Steril* [Internet] 2011;**95**:624–625.

Liao C, Kannan K. A survey of bisphenol A and other bisphenol analogues in foodstuffs from nine cities in China. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* [Internet] 2014;**31**:319–329.

Liao C, Liu F, Alomirah H, Loi VD, Mohd MA, Moon HB, Nakata H, Kannan K. Bisphenol S in urine from the United States and seven Asian countries: occurrence and human exposures. *Env Sci Technol* [Internet] 2012a;**46**:6860–6866.

Liao C, Liu F, Guo Y, Moon HB, Nakata H, Wu Q, Kannan K. Occurrence of eight bisphenol analogues in indoor dust from the United States and several Asian countries: implications for human exposure. *Env Sci Technol* [Internet] 2012b;**46**:9138–9145.

Liu X, Miao M, Zhou Z, Gao E, Chen J, Wang J, Sun F, Yuan W, Li DK. Exposure to bisphenol-A and reproductive hormones among male adults. *Env Toxicol Pharmacol* [Internet] 2015;**39**:934–941.

Mazaud-Guittot S, Nicolaz CN, Desdoits-Lethimonier C, Coiffec I, Maamar M Ben, Balaguer P, Kristensen DM, Chevrier C, Lavoué V, Poulain P, *et al.* Paracetamol, aspirin, and indomethacin induce endocrine disturbances in the human fetal testis capable of interfering with testicular descent. *J Clin Endocrinol Metab* 2013;**98**..

Meeker JD, Calafat AM, Hauser R. Urinary bisphenol A concentrations in relation to serum thyroid and reproductive hormone levels in men from an infertility clinic. *Env Sci Technol* [Internet] 2010a;**44**:1458–1463.

Meeker JD, Ehrlich S, Toth TL, Wright DL, Calafat AM, Trisini AT, Ye X, Hauser R. Semen

quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. *Reprod Toxicol* [Internet] 2010b;**30**:532–539.

Meeker JD, Yang T, Ye X, Calafat AM, Hauser R. Urinary concentrations of parabens and serum hormone levels, semen quality parameters, and sperm DNA damage. *Env Heal Perspect* [Internet] 2011;**119**:252–257.

Mendiola J, Jorgensen N, Andersson AM, Calafat AM, Ye X, Redmon JB, Drobnis EZ, Wang C, Sparks A, Thurston SW, *et al.* Are environmental levels of bisphenol a associated with reproductive function in fertile men? *Env Heal Perspect* [Internet] 2010;**118**:1286–1291.

Minguez-Alarcon L, Hauser R, Gaskins AJ. Effects of bisphenol A on male and couple reproductive health: a review. *Fertil Steril* 2016;**15**:864–870.

Muncke J. Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source? *Sci Total Env* [Internet] 2009;**407**:4549–4559.

N'Tumba-Byn T, Moison D, Lacroix M, Lecureuil C, Lesage L, Prud'homme SM, Pozzi-Gaudin S, Frydman R, Benachi A, Livera G, *et al.* Differential effects of bisphenol A and diethylstilbestrol on human, rat and mouse fetal Leydig cell function. *PLoS One* 2012;**7**:e51579.

Ohno S, Nakajin S. Determination of mRNA expression of human UDP-glucuronosyltransferases and application for localization in various human tissues by real-time reverse transcriptase-polymerase chain reaction. *Drug Metab Dispos* [Internet] 2009;**37**:32–40.

Oldring PK, Castle L, O'Mahony C, Dixon J. Estimates of dietary exposure to bisphenol A (BPA) from light metal packaging using food consumption and packaging usage data: a refined deterministic approach and a fully probabilistic (FACET) approach. *Food Addit*

Contam Part A Chem Anal Control Expo Risk Assess [Internet] 2014;**31**:466–489.

Roelofs MJ, Berg M V, Bovee TF, Piersma AH, Duursen MB. Structural bisphenol analogues differentially target steroidogenesis in murine MA-10 Leydig cells as well as the glucocorticoid receptor. *Toxicology* [Internet] 2015;**329C**:10–20.

Rosenmai AK, Dybdahl M, Pedersen M, Alice van Vugt-Lussenburg BM, Wedebye EB, Taxvig C, Vinggaard AM. Are structural analogues to bisphenol a safe alternatives? *Toxicol Sci* [Internet] 2014;**139**:35–47.

Roulet V, Denis H, Staub C, Tortorec A Le, Delaleu B, Satie AP, Patard JJ, Jégou B, Dejuq-Rainsford N. Human testis in organotypic culture: Application for basic or clinical research. *Hum Reprod* [Internet] 2006;**21**:1564–1575.

Schmidt J, Kotnik P, Trontelj J, Knez Z, Masic LP. Bioactivation of bisphenol A and its analogs (BPF, BPAF, BPZ and DMBPA) in human liver microsomes. *Toxicol Vitr* [Internet] 2013;**27**:1267–1276.

Song S, Ruan T, Wang T, Liu R, Jiang G. Distribution and preliminary exposure assessment of bisphenol AF (BPAF) in various environmental matrices around a manufacturing plant in China. *Env Sci Technol* [Internet] 2012;**46**:13136–13143.

Sungur S, Koroglu M, Ozkan A. Determination of bisphenol a migrating from canned food and beverages in markets. *Food Chem* [Internet] 2014;**142**:87–91.

Tanaka M, Nakaya S, Katayama M, Leffers H, Nozawa S, Nakazawa R, Iwamoto T, Kobayashi S. Effect of prenatal exposure to bisphenol A on the serum testosterone concentration of rats at birth. *Hum Exp Toxicol* 2006;**25**:369–373.

Teeguarden JG, Hanson-Drury S. A systematic review of Bisphenol A “low dose” studies in the context of human exposure: a case for establishing standards for reporting “low-dose” effects of chemicals. *Food Chem Toxicol* [Internet] 2013;**62**:935–948.

- Vandenberg LN. Non-monotonic dose responses in studies of endocrine disrupting chemicals: Bisphenol A as a case study. *Dose-Response* 2014;**12**:259–276.
- Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgarten FJ, Schoenfelder G. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Env Heal Perspect* [Internet] 2010;**118**:1055–1070.
- Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons W V. Human exposure to bisphenol A (BPA). *Reprod Toxicol* 2007;**24**:139–177.
- Wang L, Wu Y, Zhang W, Kannan K. Widespread occurrence and distribution of bisphenol A diglycidyl ether (BADGE) and its derivatives in human urine from the United States and China. *Env Sci Technol* [Internet] 2012;**46**:12968–12976.
- Wang L, Xue J, Kannan K. Widespread occurrence and accumulation of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives in human blood and adipose fat. *Env Sci Technol* [Internet] 2015;**49**:3150–3157.
- Yang Y, Guan J, Yin J, Shao B, Li H. Urinary levels of bisphenol analogues in residents living near a manufacturing plant in south China. *Chemosphere* [Internet] 2014;**112**:481–486.
- Ye L, Zhao B, Hu G, Chu Y, Ge R. Inhibition of human and rat testicular steroidogenic enzyme activities by bisphenol A. *Toxicol Lett* 2011;**207**:137–142.
- Zhang X, Chang H, Wiseman S, He Y, Higley E, Jones P, Wong C, Al-Khedhairi A, Giesy J, Hecker M. Bisphenol A disrupts steroidogenesis in human H295R cells. *Toxicol Sci* 2011;**121**:320–327.
- Zhou Q, Miao M, Ran M, Ding L, Bai L, Wu T, Yuan W, Gao E, Wang J, Li G, *et al.* Serum bisphenol-A concentration and sex hormone levels in men. *Fertil Steril* [Internet] 2013;**100**:478–482.
- Zhou X, Kramer JP, Calafat AM, Ye X. Automated on-line column-switching high

performance liquid chromatography isotope dilution tandem mass spectrometry method for the quantification of bisphenol A, bisphenol F, bisphenol S, and 11 other phenols in urine. *J Chromatogr B Anal Technol Biomed Life Sci* [Internet] 2014;**944**:152–156.

Zhuang W, Wu K, Wang Y, Zhu H, Deng Z, Peng L, Zhu G. Association of serum bisphenol-A concentration and male reproductive function among exposed workers. *Arch Env Contam Toxicol* [Internet] 2015;**68**:38–45.

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Figure legends

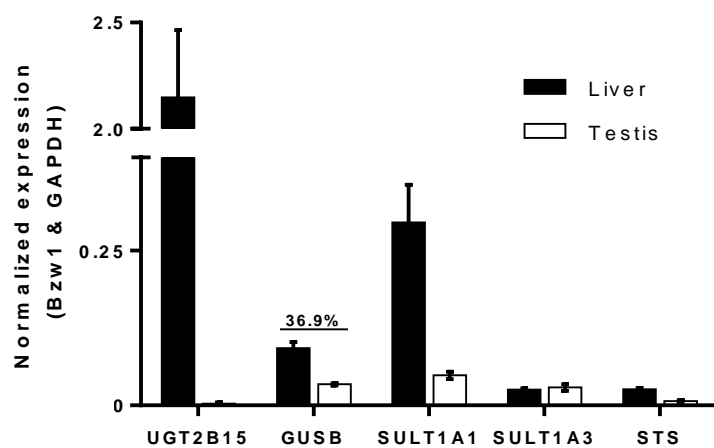


Figure 1

Figure 1: Biotransformation gene expression levels in adult human liver and testes.

Figure 1: mRNA expression levels of biotransformation enzymes.

Quantitative RT-PCRs were performed on adult human liver and testes. Each bar represents the mean \pm SEM of the fold-change in target gene expression relative to the Basic leucine zipper and W2 domains 1 (BZW1) and GAPDH reference genes. Each point represents mRNAs from three different donors. UGT2B15: UDP-glucuronosyltransferases family 2 member B15; GUSB: Beta-glucuronidase; SULT1A1: sulfotransferase family 1A member 1; SULT1A3: sulfotransferase family 1A member 3; STS: steroid sulfatase (microsomal), isozyme S. %: quantity expressed in the testis compared to the liver.

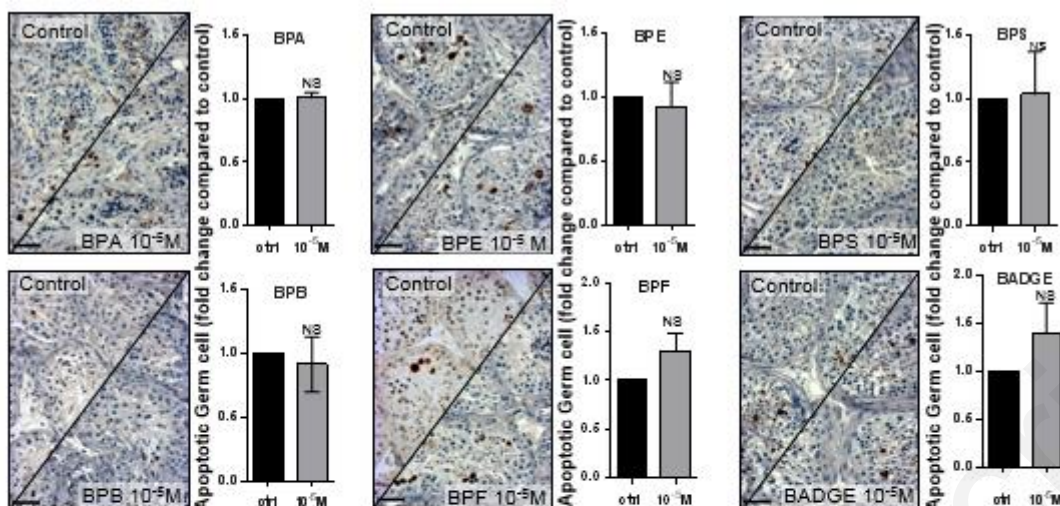


Figure 2

Fig. 2. BPA and BPA-A and morphology of the adult human testis.

Figure 2: BPA, BPA-A and histopathology.

Immunostaining of apoptotic germ cells in human testis explants cultured for 48 hr in the presence of dimethylsulphoxide (Control) or 10^{-5} M bisphenol-A(BPA) or of BPA substitutes (bisphenol E (BPE), bisphenol F (BPF), bisphenol B (BPB), bisphenol S (BPS), bisphenol A diglycidyl ether and (BADGE)) and number of apoptotic germ cells. Each micrograph shows representative areas of BPA or of BPA-A-induced morphology compared to corresponding control areas. Scale bars correspond to 50 μ m. Values are means \pm SEM of caspase-positive cells in three independent experiments from different donors. NS: Not Significant with the Mann-Whitney test.

TESTOSTERONE PRODUCTION

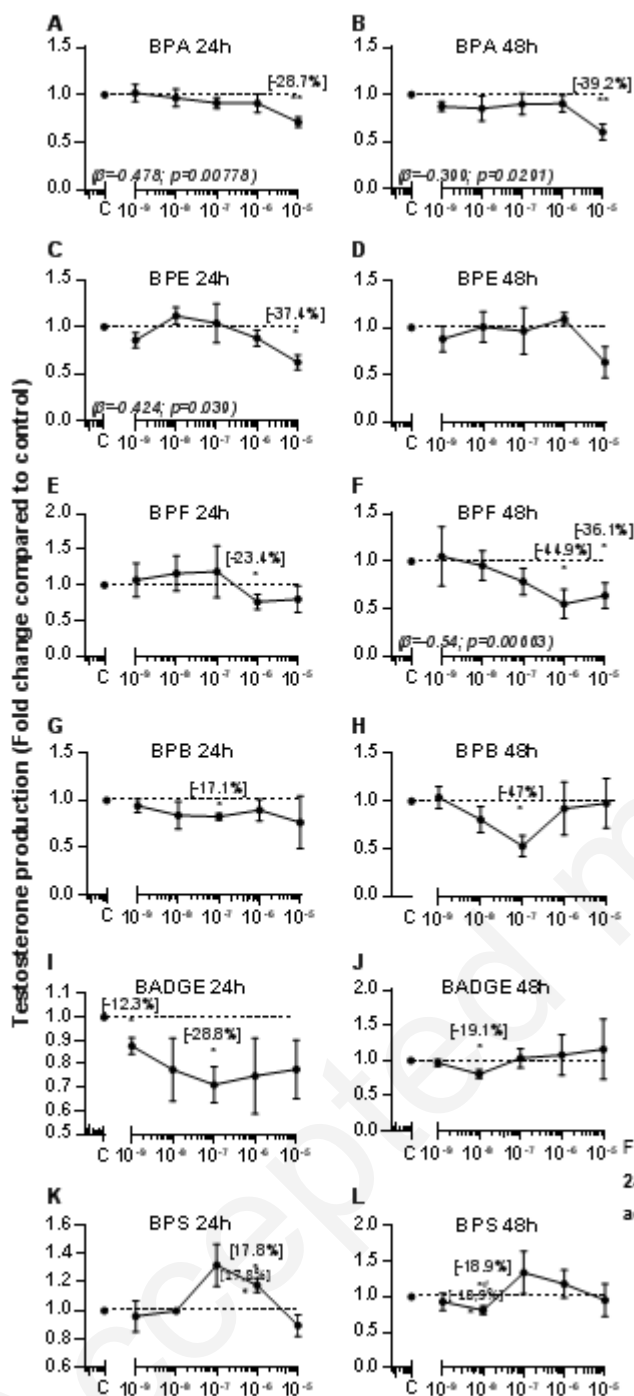


Figure 3

Figure 3: Dose effect of BPA and BPA-A exposure for 24 h (A) and 48 h (B) on testosterone production by adult human testicular explants in culture.

Figure 3: BPA, BPA-A exposure and testosterone.

The cultured explants of human testes were exposed for 24h (A) and 48h (B) to 10^{-9} - 10^{-5} M BPA or of BPE, BPF, BPB, BADGE and BPS. Values are means \pm SEM of five independent experiments from different donors. The difference from control is reported as a percentage in brackets where it is significant with the Mann-Whitney test. * $P < 0.05$. ** $P < 0.01$ *** $P < 0.001$.# $P = 0.052$.. Dose-responses were analyzed for significance with the Spearman correlations.. Slopes (β) and P values of Spearman correlations are indicated. C: control

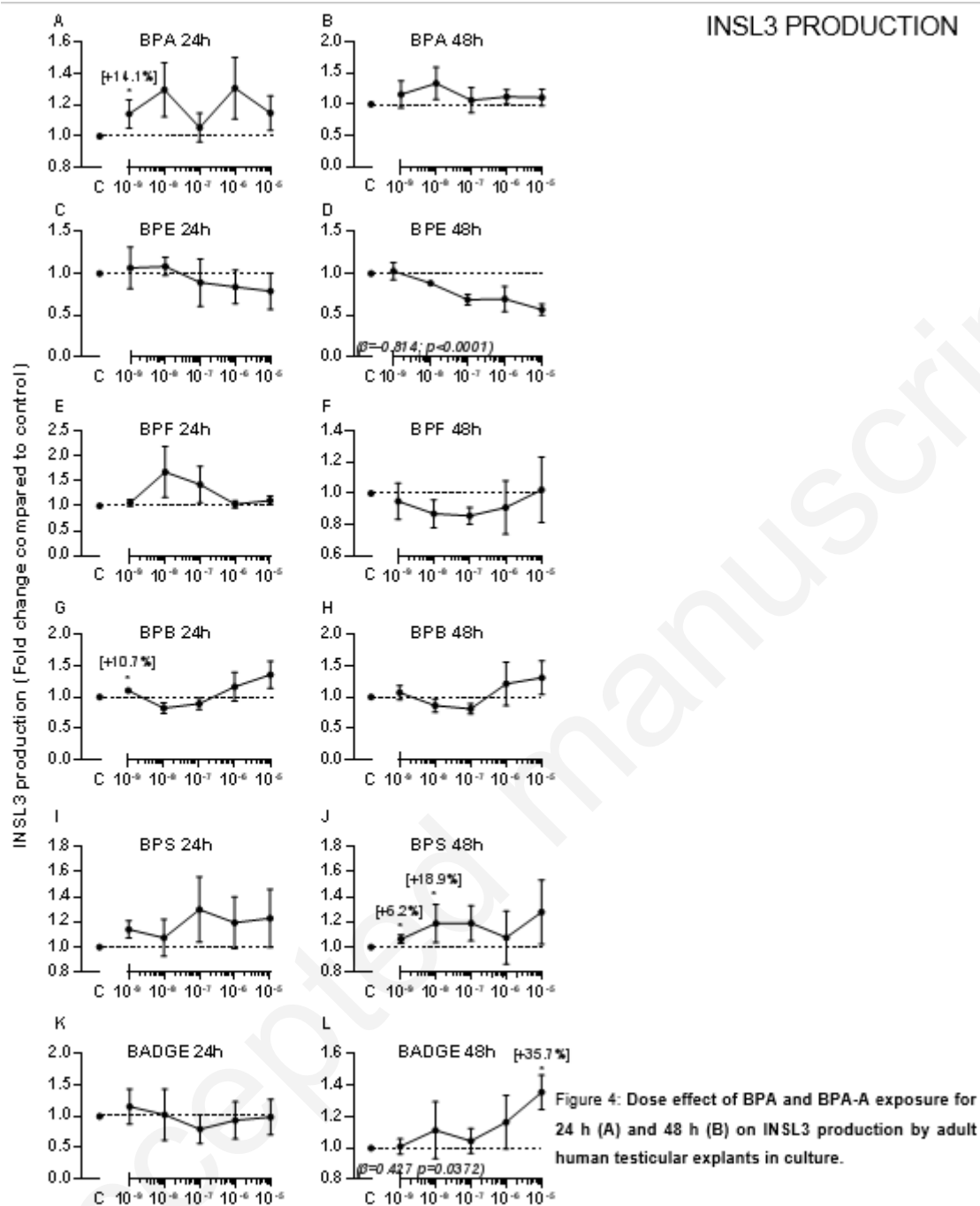


Figure 4

Figure 4: BPA, BPA-A exposure and insulin-like factor 3.

The cultured human testes explants were exposed for 24h (A) and 48h (B) to 10^{-9} - 10^{-5} M of BPA or of BPE, BPF, BPB, BADGE and BPS. Values are means \pm SEM of three independent experiments from different donors. The difference from control is given in brackets if significant with the Mann-Whitney test. * P <0.05. ** P <0.01. Dose-responses were analyzed for significance with the Spearman correlations. Slopes (β) and P values of Spearman correlation are indicated. C: control, INSL3: insulin-like factor.

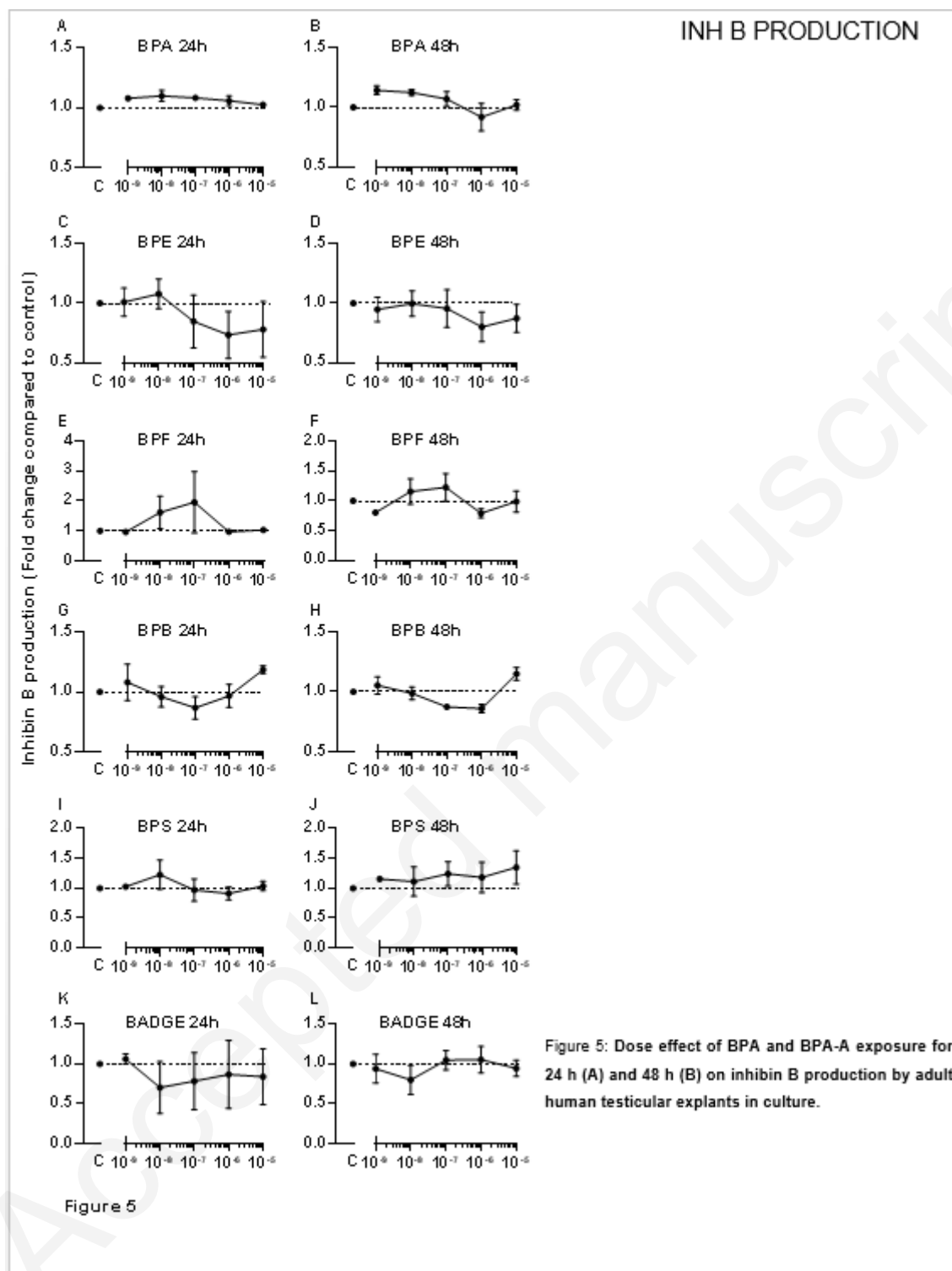


Figure 5: BPA, BPA-A exposure and inhibin B.

The cultured human testes explants were exposed for 24h (A) and 48h (B) to 10^{-9} - 10^{-5} M BPA or BPE, BPF, BPB, BADGE and BPS. Values are means \pm SEM of three independent experiments from different donors. None of the doses used at any time point caused a significant effect on inhibin B production.